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Citrus bioflavonoids dipeptidyl peptidase-4 inhibition compared with gliptin antidiabetic medications

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ABSTRACT

This study compared dipeptidyl peptidase-4 (DPP-4) inhibitory activity of citrus bioflavonoid nutraceuticals compared with three gliptins. Citrus bioflavonoid standards and three commercially available citrus bioflavonoid supplements (Thompson's Super Bioflavonoid Complex®(SB), Ethical Nutrients Bioflavonoids Plus Vitamin C®(EN), and Country Life Citrus Bioflavonoids and Rutin®(CB)) were considered in this study. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis was undertaken to identify and quantitate the citrus bioflavonoids present in each supplement. The DPP-4 inhibitory activity was determined by fluorometric assay. All of the tested individual citrus flavonoids demonstrated DPP-4 inhibitory activity, with IC_{50} values ranging from 485 μM (rutin) to 5700 μM (hesperitin and eriodictyol). Similarly, the flavonoid supplements had IC_{50} values of 16.9 mg/mL (EN), 3.44 mg/mL (SB) and 2.72 mg/mL (CB). These values compare with gliptin IC_{50} values of 0.684 μM (sitagliptin), 0.707 μM (saxagliptin) and 2.286 μM (vildagliptin). The supplement flavonoid content varied from 11.98% (CB) to 5.26% (EN) and 14.51% (SB) of tablet mass, corresponding to daily flavonoid doses of around 300, 150 and 400 mg, respectively, with CB and SB containing rutin at levels of 7.0% and 7.5% of tablet mass, respectively. While our data demonstrated that citrus bioflavonoid based supplements do possess DPP-4 inhibitory activity, they are several orders of magnitude less potent than gliptins. Further studies using higher concentrations of citrus bioflavonoids, as well as investigations into antioxidant properties which may add additional benefit are warranted.

1. Introduction

Metformin is typically considered the first line of treatment for hyperglycaemia in type 2 diabetes mellitus. However, additional treatment options include agents that specifically inhibit the enzyme dipeptidyl peptidase-4 (DPP-4) that leads to a reduction in blood glucose levels. DPP-4 is a catalyst responsible for the inactivation of incretin hormones (intestinal peptides). Increasing the activity of incretins via the use of DPP-4 inhibitors (also referred to as gliptins) improves blood glucose levels by increasing insulin sensitivity [1]. Sitagliptin, vildagliptin and saxagliptin are commonly used DPP-4 inhibitors [2]. In addition to glucose lowering effects, gliptins may also possess some cardiovascular protective effects and their use may improve beta-cell survival [2].

The DPP-4 enzyme is comprised of four structures which are homologous to the serine proteases of DPP-4; attractin, fibroblast activation protein (FAP), DPP-8 and DPP-9 [3]. The dipeptidyl peptidase family possesses a specific property of cleaving N-terminal dipeptides from polypeptides [4,5]. DPP-4 contains 766 amino acids and is a member of the S9b serine protease family [4,6]. DPP-4 is considered as a ubiquitous enzyme with activity in human epithelial cells of the intestine, kidney, liver, lung, thymus, lymph node, spleen, prostate and in activated lymphocytes and monocytes [7–9].

DPP-4 plays a key role in maintaining the secretion of insulin by

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deactivating the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose insulinotropic polypeptide (GIP). The key regions of DPP-4 are the catalytic region, cysteine-rich region and the glycosylated region while the membrane-associated domain of DPP-4 consist of a flexible segment, a transmembrane and cytoplasmic domain [10]. The DPP-4 enzyme plays a key role in regulating incretin hormones that are responsible for controlling insulin secretion. It has been previously reported that inhibition of the DPP-4 enzyme improves hyperglycaemia [11].

Flavonoids are polyphenolic plant-derived secondary metabolites that are ubiquitous in nature and are available in various flora. In many plants flavonoids primarily exist in the form of glycosides. Flavonoids can be divided on the basis of their structural characteristics into six sub-classes: flavonols, flavones, isoflavones, flavanones, anthocyanins and flavanols [12]. Citrus fruits like mandarins, tangerines, oranges, pummelos, hybrids, lemons and limes [13] are mainly tropical and subtropical fruits that contain differing amounts and types of flavonoids. Previous studies have shown that citrus bioflavonoids possess antioxidant [14], anti-inflammatory [15], anticancer [16,17], antidiabetic [18,19] and lipid lowering effects [20]. Flavanones present in grapefruit are narirutin and naringin, while hesperidin and narirutin are found in oranges and eriocitrin in lemons [21]. Commercial supplements containing citrus bioflavonoids often also contain vitamin C and additional flavonoids from other sources.

A study of berry and citrus phenolic compounds performed computationally identified bioflavonoids as potential DPP-4 inhibitors [22]. While there is increasing evidence that dietary polyphenols may influence carbohydrate metabolism at many levels [23] studies that demonstrate specific mechanisms are lacking.

The aims of this study were two-fold: to assess the DPP-4 inhibitory activity of individual citrus bioflavonoids and gliptins through *in-silico* molecular docking calculations and *in-vitro* experiments using a DPP-4 activity kit; and to analyse citrus bioflavonoid supplements for their flavonoid content, determine their inhibition of DPP-4 and thus assess their potential as natural alternatives to the gliptins for glycaemic control.

2. Materials and methods

2.1. Molecular docking of DPP-4 with different ligands

2.1.1. Ligand preparation

The structures of the citrus bioflavonoids and gliptins were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov). The structures included rutin (CID-5280805), eriocitrin (CID-83489), eriodictyol (CID-440735), naringenin (CID-439246), naringin (CID-442428), hesperidin (CID-10621), hesperetin (CID-72281), sitagliptin (CID: 4369359), anagliptin (CID: 44513473), saxagliptin (CID: 11243969), and vildagliptin (CID: 6918537).

2.1.2. Receptor preparation

The DPP-4 crystal structure (PDB ID-2ONC) was obtained from the Protein Data Bank (PDB) (**Error! Hyperlink reference not valid.**www.rcsb.org/pdb/home/home.do).

2.1.3. Molecular docking

The docking was performed using Docking Server with Autodock Vina software [24]. The docking was carried out with the default parameters: root mean square value was 2.0, population size was 150, number of evaluation was 2 500 000, number of generation created were 540 000 with 100 runs for each docking analysis. The optimal free binding energy and inhibition constant were determined for all the ligands.

2.2. Ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

2.2.1. Chemicals and reagents

All the standards and reagents were analytical grade. Eriocitrin, hesperidin, hesperetin, naringin and naringenin were purchased from Aktin Chemicals Inc (Chengdu, China). Rutin was from Sigma-Aldrich (Castle Hill, NSW, Australia).

2.2.2. UPLC-MS/MS analysis

Chromatography was performed using a Waters Acquity®H-class UPLC on a BEH C18 column (2.1 × 100 mM × 1.7 μM) (Waters Corporation, Milford, MA, USA). The mobile phase consisted of 1.0% (v/v) acetic acid (solvent A) and acetonitrile (solvent B). Elution consisted of an initial value of 100% solvent A before a gradient to 40% solvent B over 7 min. This was held for 1 min before returning to the initial conditions and re-equilibration for 3 min. The flow rate was 0.35 mL per min and the column was held at 45 °C. Injection volume was 2 μL. The UPLC was coupled to a Waters Xevo®tandem mass spectrometer (Waters Corporation). Analyses were undertaken using multiple reaction monitoring (MRM) in negative electrospray ionisation mode, performed with a capillary voltage of 2.7 kV, and individual cone voltages and collision energies for each MRM transition, as described (Table 1). The desolvation temperature was 450 °C, nebulising gas was nitrogen at 950 L/h and cone gas was nitrogen at 50 L/h. MRM transition dwell times were 0.018 s.

2.3. Dipeptidyl peptidase-4 fluorometric assay

2.3.1. Supplements and gliptins

The supplements investigated in this study were: Thompson's Super Bioflavonoid Complex®(SB) containing 500 mg citrus bioflavonoids, 500 mg vitamin C and 100 mg rutin; (Integria Health Care, Eight Mile Plains, QLD, Australia); Ethical Nutrients Bioflavonoids Plus Vitamin C®(EN) containing 500 mg citrus bioflavonoids and 500 mg vitamin C (Health World Limited, Northgate, QLD, Australia); and Citrus Bioflavonoids and Rutin®(CB) containing 900 mg citrus bioflavonoids, 100 mg rutin and 37.5 mg hesperidin (Country Life, Hauppauge, NY, USA).

The gliptin formulations investigated in this study were: Januvia®containing 100 mg of sitagliptin (Merck, Kenilworth, NJ, USA); Galvus®containing 50 mg vildagliptin (Novartis, Basel, Switzerland) and Onglyza®containing 5 mg of saxagliptin (AstraZeneca Pharma India Limited, Bengaluru, India).

2.3.2. Sample preparation

Five tablets of each supplement and three tablets of each gliptin formulation were weighed and then ground using a mortar and pestle. Each powdered supplement (100 mg) and gliptin (10 mg) were transferred into an Eppendorf tube and 1 mL of dimethyl sulfoxide (DMSO) was added. These mixtures were vortex mixed

Table 1
Quantitation MRM and electrospray ionisation parameters of each analyte.

Analytes	Rt (min)	Precursor(*m/z*)	Product(*m/z*)	Cone V	Col V
Rutin	3.65	609.4	300.2	49	36
Eriocitrin	3.67	595.4	287.2	49	22
Naringin	4.53	579.4	271.2	49	32
Hesperidin	4.71	609.4	301.2	49	24
Eriodictyol	5.87	287.2	151.1	49	15
Naringenin	6.91	271.2	151.1	49	18
Hesperetin	7.15	301.2	164.1	49	25

Rt-retention time Col-column.

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